Recommendations for microbial source tracking: Lessons from a methods comparison study

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ABSTRACT

The methods comparison study described in accompanying manuscripts demonstrated the potential value of microbial source tracking (MST) techniques, but also identified a need for method refinement. This paper provides three classes of recommendations to improve MST technology: optimization, development and evaluation. Optimization recommendations focus on library-dependent methods and include improved selection of restriction enzymes or antibiotics, better definition of appropriate library size, selection of target species and choice of statistical pattern-matching algorithms. Methods development recommendations focus on identifying new genomic targets and quantification procedures for library-independent methods. Longer-term methods development recommendations include integration of microarrays and other direct pathogen detection technology with MST. Studies defining host specificity and population dynamics should aid selection of target species during methods development. Evaluation recommendations include enhancements that should be incorporated into future methods comparison studies, along with studies to assess the value of MST results for risk characterization.

Key words | coliform, fecal contamination, microbial source tracking, water quality

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INTRODUCTION

Microbiological source tracking (MST) techniques have been used in numerous locations to successfully identify the dominant source of fecal contamination. In Holmans Creek, Virginia, antibiotic resistance analysis (ARA) identified humans as the primary fecal source, and resulting septic improvements (pump-outs, upgrades, etc.) caused contamination levels to decline (Dart & Wiggins 2003). F^+RNA coliphage typing was used to demonstrate that birds were the major contributors of fecal contamination to a municipal reservoir in New York (Alderisio *et al.* 1996), leading to a successful bird deterrent programme. In Avalon, California, Boehm *et al.* (2003) used human-specific markers for *Bacteroides/Prevotella* and enterovirus to correctly identify human sewage as the main source of fecal pollution.

Despite a number of successful applications, MST techniques are still under development. The accompanying papers of this issue describe an evaluation study in which MST methods were collectively found to be useful, but the study also demonstrated the need for method refinement (Griffith *et al.* 2003). Library-based

methods consistently identified the dominant source in blind seeded samples, but frequently identified sources that were not present (Harwood *et al.* 2003; Myoda *et al.* 2003). Non-quantitative methods resulted in fewer false positives, but the highest correct classification rates were produced by methods that could identify only a limited number of sources. PCR of host-specific *Bacteroides-Prevotella* reliably identified samples seeded with human or cow wastes, but was not yet able to identify other sources (Field *et al.* 2003*a*). Virus methods consistently detected human contamination in sewage-seeded samples, but are not designed to identify samples containing fecal contamination from individual, healthy humans (Noble *et al.* 2003).

This paper incorporates results from the evaluation study to develop recommendations for future refinement of MST methods. Three categories of recommendations are provided. The first is optimization, in which existing methods are enhanced through improved understanding of how they work and which technique options are most effective. The second involves development of new methods, or marked modifications to existing methods. The third is method evaluation, as researchers in the field need to conduct additional and even more challenging studies than were undertaken in this evaluation study.

METHODS OPTIMIZATION

Library-based MST methods are currently the mostly widely used, but researchers are quite varied in their application of these methods (Harwood *et al.* 2003; Myoda *et al.* 2003). Differences in implementation between practitioners are beneficial during the methods development phase, as a variety of options for improving method efficiency are still being explored. As method development advances, it becomes more important to assess which of these variations provides the greatest discriminatory power and accuracy. At even later stages of development, methods need to be standardized so that all practitioners can produce equivalent results. The culmination of optimization and standardization is methods approval by the EPA and/or the American Public Health Association, Water Environment Federation and the American Water Works Association (*Standard Methods for the Examination of Water and Wastewater*).

The first method optimization need is to settle on a method for eliciting a response that will be compared between a library and a sample. Practitioners of antibiotic resistance analysis (ARA) differ considerably in the number and concentrations of antibiotics used. Clearly, some combinations are more effective than others, but it is not yet clear which is best. Analogues for genotypic methods include selection of primers and restriction enzymes.

One part of optimizing the response is determining which bacterial species provides the greatest discrimination. In the accompanying evaluation study, ARA of *Enterococcus* was found to produce better results than that of *E. coli* (Harwood *et al.* 2003), though this could also reflect differences in antibiotic panels used or be specific to the samples tested. These kinds of confounding factors will need to be studied in a controlled setting before there is consensus on optimal methods for standardization.

Another important optimization need is library development. One of the biggest questions is, what library size constitutes a valid and cost-efficient approach? Practitioners are presently making decisions about library size based on logistical and cost constraints and/or on ad hoc rules of thumb developed through practical experience. No studies to date have rigorously evaluated the minimum or optimal library size, though additional sampling may not be necessary to address this issue. Currently, the largest known library of ribotyping patterns from human sources contains over 3,800 *E. coli* isolates, of which over 950 patterns are unique (Mansour Samadpour, personal communication). Statistical analyses of libraries such as this one, if properly analysed, could provide useful information about optimal library size.

The amount of necessary library development for a particular study is also dependent on the temporal and geographic stability of libraries. High variability in either parameter could restrict the use of many methods to local venues because of the time and cost constraints inherent in constructing suitable libraries. A few studies have suggested that geographic variability can be high and application of library-based methods should be locally derived

(Hartel *et al.* 2002; Wiggins *et al.* 2003), though further evaluation is warranted. The geographic applicability of a library is likely to be affected by a number of factors, including hydrology and animal migration patterns. Temporal variability has been even less well studied. Seasonal dietary shifts or changes in other selective pressures could lead to unstable libraries. At least one study suggested that libraries are subject to marked temporal variations (Jenkins *et al.* 2003), while another found a library to be stable for at least one year (Wiggins *et al.* 2003). Ultimately, the selection of which marker type (e.g. phenotypic vs. genotypic) is optimal for widespread application may depend on which has the most stable host-specific relationship.

The future of MST may feature 'super libraries', which include profiles for bacteria that could be used over large geographical areas. It is possible that insufficient sampling could explain much of the observed temporal and geographical variations of microbial subspecies. It should theoretically be possible to create a large library representative of a microbial population that would only require updates for temporal population changes. The challenge is for researchers to identify a predominantly host-specific microbe and to apply a method that is adequately, but not overly discriminatory. This is a complex undertaking, but establishment of a super library and central database similar in format to the Center for Disease Control's (CDC) PulseNet molecular subtyping network for food borne disease surveillance could make MST more routine and widely available. Of course, this can only happen after methods have become more standardized.

Once a response is elicited, researchers must match the resulting patterns to that from a library, and researchers are presently using a variety of algorithms to accomplish this. Some of these algorithms require a very close, or even exact, match to classify an isolate, but do so at the cost of eliminating much of the data. Other algorithms classify every isolate, regardless of how well the pattern matches a library strain. One of the researchers who produced relatively high accuracy in the methods comparison study relied on a 1:1 matching (Myoda *et al.* 2003), but Ritter *et al.* (2003) found that the best band-matching algorithm can differ between methods. There are certainly opportunities for further research in this area, perhaps through simulations using existing library data.

A final part of optimization is concerned with selecting appropriate combinations of methods. No single method was found to be absolutely effective during the methods comparison study and a combination of methods might serve to increase a manager's confidence in decision-making based on MST results. In general, host-specific PCR and the virus methods were effective at determining whether a human source was present, but they are not yet quantitative. In contrast, box-PCR, ribotyping and pulsed-field gel electrophoresis (PFGE) are quantitative and provided a reasonable assessment of dominant source with some of the lowest false positive rates, but they occasionally identified low to moderate amounts of human source material when it was not present in the sample. The financial implications of incorrectly identifying the presence of human source material and taking management actions in response can be serious. Using the non-quantitative methods to confirm the presence of a human source could allow managers to avoid potentially costly incorrect decisions.

The need for, and manner of use of, method combinations depends on the application. There is extra expense associated with using multiple methods and this cost must be considered in context of costs for an incorrect decision. For instance, the cost implications are low and multiple methods may not be warranted in a rural, non-contact stream where the source is believed to be animal runoff. In contrast, bacterial problems on Huntington Beach, California, were postulated to be from either urban runoff or an offshore wastewater treatment outfall plume that was reaching shore (Boehm *et al.* 2002), with a several hundred million dollar cost for addressing the latter possibility. In the latter case, using several methods in combination would be financially prudent.

METHODS DEVELOPMENT

The most frequent applications of MST at present use library-dependent methods, but the future is more likely to

focus on library-independent approaches. Library-independent methods offer many advantages. They have the potential to be considerably cheaper and faster because they do not require the investment in library development. They also have the potential for greater accuracy, since they focus on a specific trait rather than attempting to pattern-match a large number of isolates, some of which may be transient among species. PCR primers, designed to distinguish human and ruminant *Bacteroides–Prevotella*, achieved 92% accuracy for presence/absence of both human and cow wastes during the methods comparison study (Field *et al.* 2003*a*). Gene-specific primers for a larger number of animals are under development and could expand the potential applicability of this approach (Field *et al.* 2003*b*).

Advances in molecular detection methods are likely to bring about increased opportunities for direct pathogen measurement, which could enhance specificity to human sources. Techniques such as q-PCR or genetic arrays that target pathogens may eventually replace the indicator organism approach and provide a higher level of source certainty. Nucleotide sequencing may also be added to the MST toolbox. Sequencing is becoming cost-efficient, making direct comparisons between the nucleic acids of environmental and source isolates more practical. Identification of uniquely related isolates (e.g. with shared point mutations) could provide compelling evidence of pollution sources for discrete problem areas.

The biggest impediment to method development is our poor understanding of population genetics and host specificity of MST microorganisms. Approaches to MST often assume that microbial subtypes are specific to a particular host species. For bacteria, this assumption is based on a clonal replication paradigm and further assumes co-evolution of intestinal flora and hosts. The genetics and ecology of intestinal flora, however, are unlikely to be this simplistic. Mutation, recombination, migration, selection and drift may affect genetic diversity of intestinal bacteria. Genetic diversity within a single host varies with time, diet and antibiotic use. For example, Caugant *et al.* (1981) identified 53 distinct electrophoretic types of *E. coli* among 550 isolates sampled from a single human host over an 11-month period using multiple locus enzyme electrophoresis (MLEE). Two types were identified as 'residents', appearing repeatedly over time and accounting for 343 (62%) of the tested isolates. The remaining types were identified as 'transients', appearing for only one or a few days.

Studies quantifying the genetic diversity of *E. coli* and other enteric bacteria have found that the host species is only one, possibly minor, factor related to genetic diversity (Ochman *et al.* 1983; Souza *et al.* 1999; Gordon & Lee 1999). Multilocus enzyme electrophoresis (MLEE) was used for typing in these studies. MLEE analyses enzymes, while most of the molecular marker or genetic fingerprinting methods used for MST directly analyse nucleic acids. Estimates of genetic diversity based on enzyme electrophoresis have been correlated with those based on DNA reassociation tests (Ochman *et al.* 1983). However, further studies are warranted to determine if the chromosomal genomes of bacterial populations are influenced by factors such as habitat and time to the same extent as expressed proteins. Some of these basic genetics and ecology questions may be answered soon by data generated using multilocus sequence typing (MLST), nucleotide sequencing or MST fingerprinting techniques. Increased communication between water quality microbiologists performing MST research and microbial population biologists evaluating genetic diversity of enteric microorganisms would facilitate this process.

Microorganisms currently used for MST include the traditional coliform group, including *Escherichia coli*, the fecal streptococci and enterococci, species of *Bacteroides*, bacteriophage, and enteric entero- and adenoviruses. However, it is estimated that a minimum of 10^{14} bacteria populate the human intestinal tract, including as many as 500 different bacterial species. Future approaches to MST may need to incorporate species other than those currently in use, particularly if the currently used species are found to have poor host specificity. Candidate intestinal flora, based on ecological considerations, may include *Staphylococcus aureus* or *epidermidis* (found in low numbers in the colon), species of *Proteus*, *Campylobacter jejuni*, *Rhodococcus coprophilus* (an aerobic, nocardioform actinomycete) and *Selenomonas ruminantium*. There are 10^3 more obligate anaerobic bacteria in the lower intestine than facultative or aerobic microorganisms. Anaerobes, including those in the genera

Fusobacterium, *Veillonella* and *Clostridium*, could also be reconsidered. While traditional culture may not be a practical option, knowledge of the molecular genetics of these bacteria could make them accessible for MST.

In addition to host specificity, population genetics studies are needed to examine changes in microbial populations entering the environment from intestinal habitats. This transition may be dominated by physiological adjustments, which could result in a relatively stable genetic population structure, or by natural selection, which could result in significant changes in population genetics (Savageau 1983; Gordon *et al.* 2002). A limited number of studies have examined population differences during habitat transition using MLEE (Whittam 1989; Gordon *et al.* 2002). These studies have revealed that the genetic composition of *E. coli* populations can change dramatically from intestinal to environmental habitats, suggesting that natural selection dominates during this transition. These studies also demonstrated that a percentage of bacterial subtypes associated with particular sources could be identified in environmental samples. Adaptation, in the form of altered gene expression, has also been observed for *E. coli* in a simulated aquatic environment (Espinosa-Urgel & Kolter 1998).

METHODS EVALUATION

MST methods have undergone limited evaluation testing, which needs to be expanded. For library-based methods, most previous testing has been fairly simplistic, focusing primarily on blind testing of isolate identification against a known library of the same isolates. The methods comparison study described in accompanying papers expanded on this by assessing whether fecal sources could be accurately identified when placed in an aqueous matrix in various combinations. None of the MST methods was entirely effective at identifying dominant sources in those samples. Further testing of this type as methods improve is clearly necessary until an accurate answer can be consistently produced.

The methods comparison study was relatively simplistic and needs to be expanded before managers can apply these methods with full confidence. For instance, all of the fecal samples used to construct the test samples in this study were available to the investigators as library material, whereas in a typical application the library must be extrapolated from a small percentage of animals in the watershed that might have contributed fecal material to the water. The next level of validation needs to incorporate evaluation of this extrapolation. One possible scenario would be to conduct a similar study, but create blind aqueous test samples using fecal material from different animals from those provided as library material.

An even more challenging evaluation would be to draw the blind samples from relatively simple watersheds dominated by a single source. For example, samples could be drawn from runoff directly below a dairy farm, a heron rookery or a leaking septic system. This approach would be limited to identifying the dominant source, since other sources would probably also be present, but it would enhance the test by including soil bacteria, potential mixtures of sources and other real-world interferences that cannot be accurately mimicked in laboratory-created samples. This field validation approach has been used for some individual methods (Whitlock *et al.* 2002), but has not been applied to multiple methods simultaneously.

While MST tools are presently used primarily for guiding managers towards the most appropriate source reduction strategy, these tools may also have value in risk characterization. Public health managers presently use enterococcus and fecal coliform density as indicators of human sewage, issuing warnings or closing recreational beaches when standards are exceeded. However, animal fecal material, which is believed to have a lower human health risk than human fecal material, also contain the same indicator bacteria. Many states incorporate crude MST approaches, such as measurement of the total:fecal coliform ratio (Haile *et al.* 1999), measurement of *Clostridium perfringens* (Fujioka 2001) or direct visual observation to look for leaking sewer lines, as supplemental monitoring efforts to modify the need for or nature of their health warnings.

Genotypic, phenotypic and direct pathogen MST techniques can potentially improve upon these practices, but their expanded use in a risk characterization framework requires additional types of validation studies. Most

importantly, MST methods need to be incorporated into epidemiological studies. Human fecal material contains a greater number and density of human pathogens than animal fecal material, but most enteric pathogens are harboured by a variety of animals, particularly mammals (Scott *et al.* 2002). Knowing the relative risk from human, mammal and other animal fecal sources is a necessary precursor to using MST in routine monitoring/health warning systems.

CONCLUSIONS

Each MST method has been found to have advantages and limitations during field applications and in the methods comparison study, but more research is needed to optimize and evaluate the various techniques. It is unlikely that any one method will evolve as a standard over the next few years. Instead, method(s) need to be carefully selected depending on study objectives. Combining multiple methods from the MST toolbox may also provide a viable option to assist mitigation of water quality problems. The future of MST is likely to be different from the present. Use of library-independent methods is likely to increase as markers are developed and validated, and organisms chosen for MST may change depending on host specificity.

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